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Identification of an essential sequence for dihydroceramide C-4 hydroxylase activity of mouse DES2

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Abstract Although the amino acid sequences of mouse DES1 (MDES1) and DES2 (MDES2) have 63% sequence identity, their enzymatic characteristics are quite different. MDES1 exhibits high dihydroceramide Δ^4-desaturase activity and very low C-4 hydroxylase activity, while MDES2 is similarly active as both a dihydroceramide Δ^4-desaturase and a C-4 hydroxylase. We constructed several chimeras of MDES1 and MDES2 and identified a region important for C-4 hydroxylase activity in MDES2. This region contains the sequence XAFGY (X = T or A or V; Y = T or N) and occurs on the C-terminal side of the first His-box of MDES2. We confirmed the conservation of this region in DES2 family members sequenced from humans, pigs, rats, chickens, zebrafish, and *Xenopus*.

Keywords: C-4 hydroxylase; Δ^4 desaturase; DES2; DES1

1. Introduction

The Δ^4-desaturation of dihydroceramide is an important step in the formation of ceramide [1–5]. This enzymatic reaction is carried out by proteins of the DES1 family, which have been identified as homologs of the *Drosophila melanogaster* degenerative spermatocyte gene-1 (*des-1*) [6–9]. Ternes et al. reported that another *des-1* homolog, DES2 of mouse and human, has sphingolipid C-4 hydroxylase and Δ^4-desaturase activities as a bifunctional enzyme. We performed an in vitro assay of a homogenate of COS-7 cells transfected with mouse DES2 (MDES2) cDNA using *N*-octanoyl-sphinganine as a substrate to show that MDES2 is a dihydroceramide C-4 hydroxylase. MDES2 is responsible for the biosynthesis of enriched phytosphingoglycolipids in the microvillous membranes of mouse intestinal epithelial cells [10].

There is 63% identity between the amino acid sequences of MDES1 and 2. MDES1 exhibits high dihydroceramide Δ^4-desaturase and very low C-4 hydroxylase activities, while MDES2 exhibits moderate Δ^4-desaturase and C-4 hydroxylase activities. MDES1 and 2 contain three highly conserved histidine-rich sequences (His-boxes) and these regions are essential for catalytic activities [11]. By constructing several chimeras of MDES1 and 2, we identified an XAFGY (X = T or A or V; Y = T or N) sequence which is located on the C-terminal side of the first His-box of MDES2 and forms a region important for C-4 hydroxylase activity. The same five amino acid residues are conserved in the DES2 family from humans, pigs, rats, chickens, zebrafish, and *Xenopus*.

2. Materials and methods

2.1. Cell culture, transfection, enzyme assay, and immunoblotting

FLAG-tagged chimeric MDESs were transfected into COS-7 cells and the enzyme activity of each homogenate was measured as previously described [10]. Immunoblotting was performed as previously described [10] using an anti-FLAG antibody, M2 (Sigma, St. Louis, MO, USA). Enzyme activities of the FLAG-tagged MDESs were normalized to FLAG concentrations determined by immunoblotting with the anti-FLAG antibody.

2.2. Construction of chimeric MDESs

FLAG-tagged chimeric MDESs were prepared by PCR using overlapping primers [12]. MDES21 was prepared as follows. First, two PCR fragments were amplified, one by using the MDES2F primer and the overlapping primer MDES21X′ to generate an N-terminal side fragment from MDES2, and the other by using the overlapping primer MDES21X and the MDES1R primer to generate a C-terminal side fragment from MDES1 (Table 1). After both PCR products were mixed and annealed, another PCR was performed using the MDES2F or MDES1R primer. MDES12 was prepared from MDES1 and MDES2 using the overlapping primers MDES12X and X′. Then, MDES212 was prepared from MDES21 and MDES2 by an additional PCR using the overlapping primers MDES212X and X′. MDES121 was prepared from MDES21i, which had been prepared from MDES1 and MDES2 using the overlapping primers MDES21iX and X′, and MDES1 by an additional PCR using the overlapping primers MDES121X and X′. MDES212-a1 was prepared from MDES2 and MDES212 by an additional PCR using the overlapping primers MDES212-a1X and X′; MDES212-a2 was prepared from MDES2 and MDES212 with the overlapping primers MDES212-a2X and X′; and MDES212-a3 was prepared from MDES212 and MDES212-a2 using the overlapping primers MDES212-a3X and X′. The resulting PCR products were cloned into a pCRII-TOPO vector (Invitrogen) and the inserted DNA sequences were analyzed with an ABI PRISM 3100 sequencer. Chimeric MDES DNA fragments were cloned into the mammalian expression vector pME18S-FLAG, which carries a FLAG peptide and a strong chimeric promoter SR-α [13].

2.3. Protein sequence comparisons

Potential sequence homologs were identified in GenBank by BLAST searches and included DES1 and DES2 family from rat, chicken, pig, zebrafish, and *Xenopus*. Sequences were aligned using the CLUSTAL W program and neighbor-joining phylogenetic trees were constructed [14,15].

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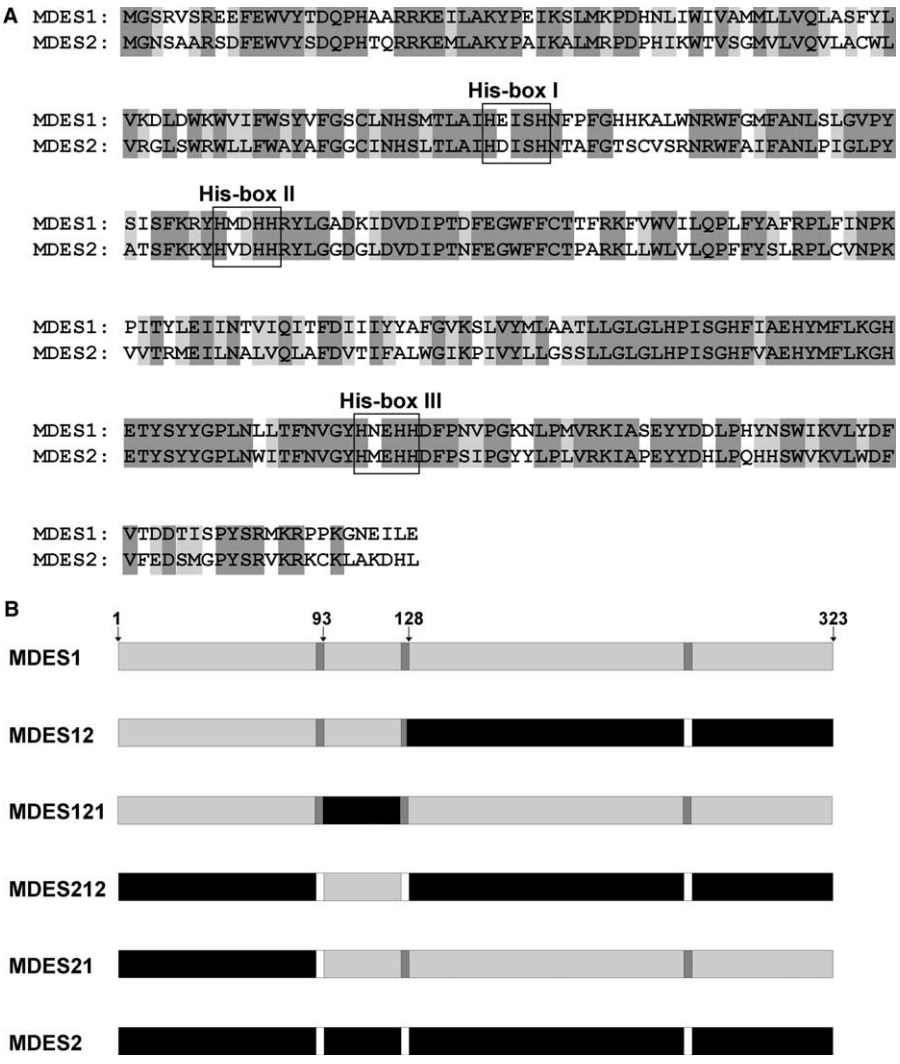

Fig. 1. Structures of MDES1 and 2, and their chimeras. (A) Comparison of the amino acid sequences of MDES1 and 2. The dark gray boxes indicate identical residues and the light gray boxes show amino acid similarity. The three His-boxes are indicated. (B) Structures of MDES1, 12, 121, 212, 21, and 2. The light gray boxes indicate amino acid residues in MDES1 and the dark gray boxes indicate MDES1 His-boxes. The black boxes indicate amino acid residues in MDES2 and the white boxes indicate MDES2 His-boxes. The numbers indicate the number of amino acid residues from the N-terminal.

3. Results

3.1. Chimeras of MDES1 and 2 did not have Δ^4-desaturase or C-4 hydroxylase activities

Although MDES1 and 2 have 63% amino acid sequence identity (Fig. 1A), their enzymatic characteristics are different. MDES1 exhibits high dihydroceramide Δ^4-desaturase and very low C-4 hydroxylase activities, with a specific activity ratio of 52.5:1 [10]. MDES2 is active at similar levels as both a desaturase and a hydroxylase, with a ratio of 2.4:1 [10]. We constructed several FLAG-tagged chimeric MDESs (as shown in Fig. 1B) and determined their enzymatic activities. MDES12, 121, 212, and 21 exhibited very low Δ^4-desaturase and C-4 hydroxylase activities, similar to the endogenous activities of host COS-7 cells (data not shown). Although MDES212 shared 95% amino acid sequence identity with MDES2 (Figs. 1B, 2A and B), and MDES121 shared 95% identity with MDES1 (Fig. 1B), both MDES212 and 121 exhibited very low Δ^4-desaturase and C-4 hydroxylase activities (data not shown).

3.2. An important region for C-4 hydroxylase activity located on the C-terminal side of the first His-box of MDES2

The replacement of a short 34-amino acid region of MDES212, between the first and second His-boxes, abolished both enzyme activities. Modifications were made to the MDES2 sequence in this region, as shown in Fig. 2A and B. MDES212-a1 exhibited recovered C-4 hydroxylase activity despite being different by just two amino acid residues from MDES212. The further replacement of 13 amino acid residues produced MDES212-a2, which is just one amino acid residue different from MDES2 (R or K); as expected, this molecule exhibited restored hydroxylase activity. However, the replacement of five amino acid residues at the C-terminal flanking sequence of the first His-box (from TAFGTSC of MDES2 to FPFGHHK of MDES1: MDES212-a3) reduced C4 hydroxylase activity to the activity level of MDES212. The Δ^4-desaturase activities of MDES212-a1, 2, and 3 were the same as those of MDES2 and MDES212 (data not shown). Based on these results, we conclude that these seven amino

Table 1
Primer sequences

Primer	Sequence
MDES1F	5′-CCTCGAGGCCATGGGTAGCCGCGTGTCC-3′
MDES1R	5′-GATATTTACTCCAGAATCTCGTTCCC-3′
MDES2F	5′-CCTCGAGGCCATGGGTAATAGCGCGGCCC-3′
MDES2R	5′-GGCTCACAGGTGGTCCTTCGCCAGCTTA-3′
MDES21X′	5′-GCCAAAGGGGAAATTGTGCGAGATGTCATGGAT-3′
MDES21X	5′-CATGACATCTCGCACAATTTCCCCTTTGGCCAC-3′
MDES12X′	5′-TCCACCCAGGTAACGGTGGTGATCCATGTGGTA-3′
MDES12X	5′-CACATGGATCACCACCGTTACCTGGGTGGAGAC-3′
MDES212X′	5′-ATGGTGGTCCACATGGTATCTCTTGAAGGAAAT-3′
MDES212X	5′-TCCTTCAAGAGATACCATGTGGACCACCATCGT-3′
MDES21iX′	5′-GTGGTGATCCATGTGGTACTTTTTGAAGGATG-TAGC-3′
MDES21iX	5′-TCCTTCAAAAAGTACCACATGGATCACCACCGG-TAC-3′
MDES121X′	5′-GCCAAAGGCAGTATTGTGGGAAATCTCATGGA-TAGC-3′
MDES121X	5′-CATGAGATTTCCCACAATACTGCCTTTGGCAC-AAGT-3′
MDES212-a1X′	5′-CAGGGCCTTGTGGTGGCCAAAGGCAGTATTG-TGCGA-3′
MDES212-a1X	5′-AATACTGCCTTTGGCCACCACAAGGCCCTGTG-GAAC-3′
MDES212-a2X′	5′-GTATCTCTTGAAGGATGTAGCGTAAGGTAGGC-CAAT-3′
MDES212-a2X	5′-CTACCTTACGCTACATCCTTCAAGAGATAC-CATGTG-3′
MDES212-a3X′	5′-GCGGTTTCGGGAGACCTTGTGGTGGCCAAAGGG-GAA-3′
MDES212-a3X	5′-TTTGGCCACCACAAGGTCTCCC-GAAACCGCTGGTTT-3′

acid residues, TAFGTSC, are essential for C-4 hydroxylase activity.

3.3. Conservation of the C-4 hydroxylase region among DES2 gene families

DES1 family homologs for *D. melanogaster des-1* have already been identified in many species including plants such as *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Schizosaccharomyces pombe*, *Candida albicans*, *Neurospora crassa*, and *Toxoplasma gondii*, and the animals *Caenorhabditis elegans*, rat, mouse, and human [6]. DES2 families have also been reported from mouse and human [6,16]. We have found DES2 family candidates in pig, rat, chicken, zebrafish, and *Xenopus*, and DES1 family candidates in pig, rat, chicken, and zebrafish by searching GenBank using the BLAST algorithm (Fig. 3).

The sequence TAFGTSC appears to be essential for C-4 hydroxylase activity in MDES2 (Fig. 2A). Therefore, we were interested in whether this region is conserved in other DES2 family genes. We compared DES2 sequences from human, pig, rat, chick, zebrafish, and *Xenopus* and have confirmed that DES2 family members have five amino acid residues, XAFGY (X = T or A or V; Y = T or N), in common in this region (Fig. 3A).

Although we did not confirm the Δ^4-desaturase and C-4 hydroxylase activities of rat, chick, zebrafish, and *Xenopus* DES1 and DES2 candidates, these sequences were classified as DES1 and DES2 families by phylogenetic analyses (Fig. 4). In support of our phylogenetic tree, Uchida et al. [17] reported that zebrafish DES1 had high Δ^4-desaturase activity and that DES2 had Δ^4-desaturase and C-4 hydroxylase activities.

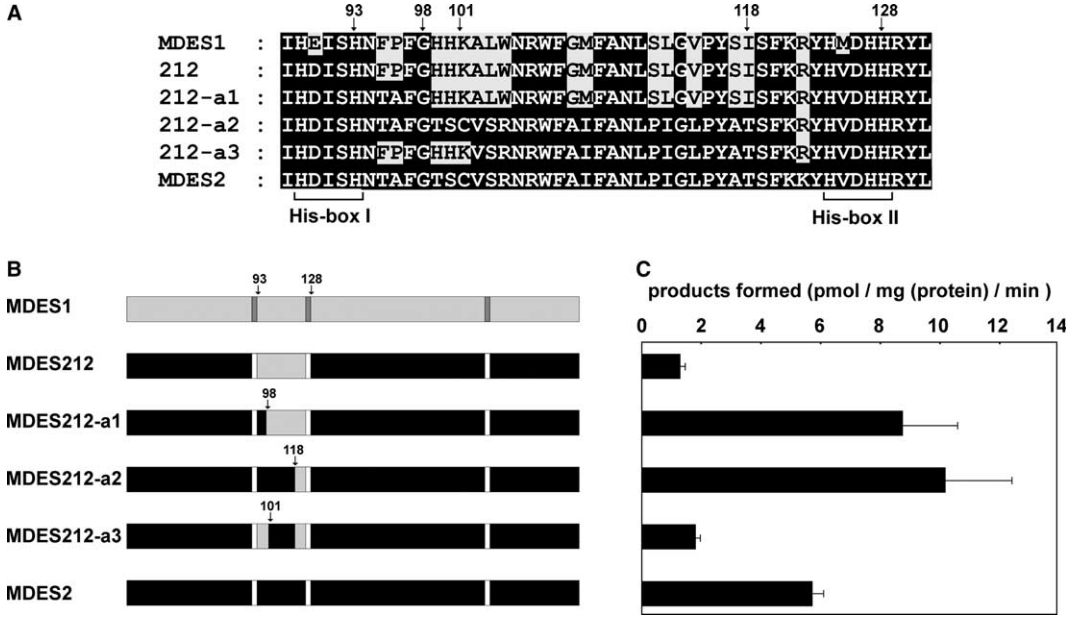

Fig. 2. The C-4 hydroxylase activities of MDES2 and its chimeras. (A) The amino acid sequences of MDES1, 212, 212-a1, 212-a2, 212-a3, and 2. The black letters with a light gray background indicate amino acid residues identical to those in MDES1, and the white letters with a black background indicate amino acid residues identical to those in MDES2. The His-boxes are indicated. The numbers indicate the number of amino acid residues from the N-terminal. (B) Structure of MDES1, 212, 212-a1, 212-a2, 212-a3, and 2. The light gray boxes indicate amino acid residues in MDES1 and the dark gray boxes indicate MDES1 His-boxes. The black boxes indicate amino acid residues in MDES2 and the white boxes indicate MDES2 His-boxes. The numbers indicate the number of amino acid residues from the N-terminal. To construct MDES212-a1, amino acid residues 94–98 in MDES212 were replaced with those for MDES2. To construct MDES212-a2, amino acid residues 94–118 in MDES212 were replaced with those for MDES2. To construct MDES212-a3, amino acid residues 94–101 in MDES212-a2 were replaced with those for MDES1. (C) The C-4 hydroxylase-specific activities of MDES212, 212-a1, 212-a2, 212-a3, and 2. The C-4 hydroxylase activity is expressed as the mean ± S.E.M. ($n = 3$).

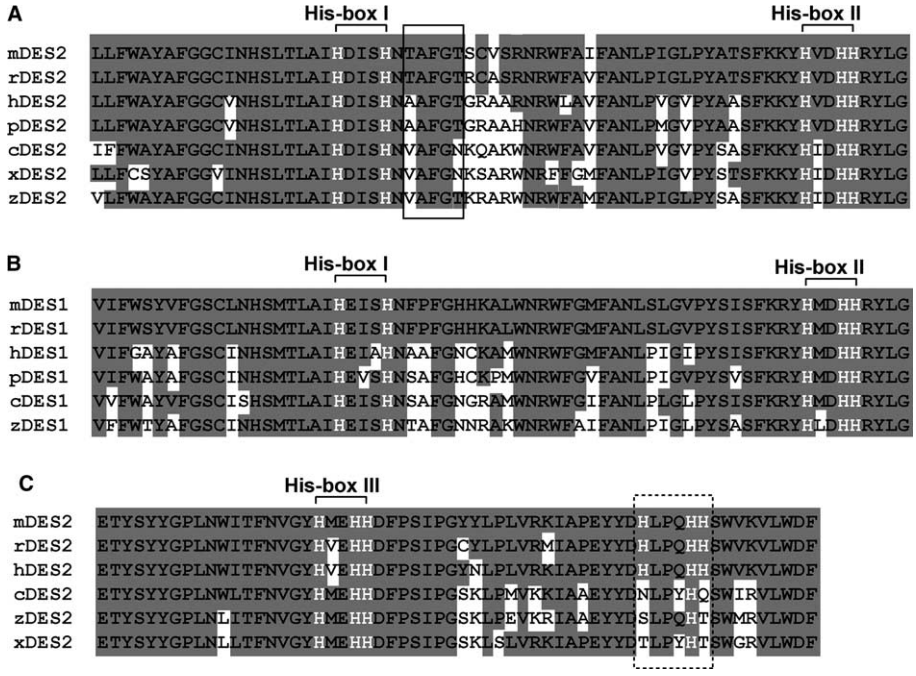

Fig. 3. Comparison of the amino acid sequences of the DES1 and 2 families. (A) Comparison of the DES2 families near the first and second His-boxes. The gray boxes indicate identical residues. The His-boxes are indicated. Solid lines enclose the XAFGY (X = T or A or V; Y = T or N) motif. (B) Comparison of the DES1 families near the first and second His-boxes. The gray boxes indicate identical residues. (C) Comparison of the DES2 families near the third His-box. The gray boxes indicate identical residues and broken lines enclose the putative fourth His-box.

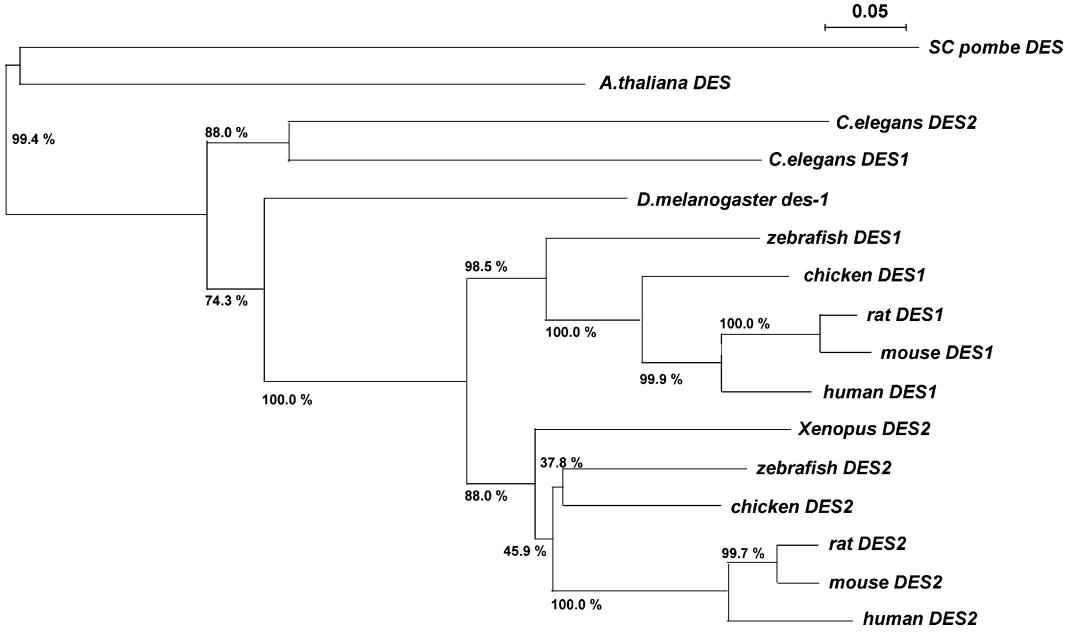

Fig. 4. The phylogenetic tree of DES1 and DES2. The tree was constructed from 16 amino acid sequences using the neighbor-joining method. The branch lengths are proportional to the relative phylogenetic distances between the proteins.

4. Discussion

Our results and those of Ternes et al. show that DES family proteins are bifunctional enzymes [6,10]. DES1 exhibits high Δ^4-desaturase and very low C-4 hydroxylase activities, while DES2 exhibits moderate Δ^4-desaturase and C-4 hydroxylase activities. MDES2 is the dihydroceramide C-4 hydroxylase responsible for the biosynthesis of enriched phytosphingoglycolipids in the microvillous membranes of intestinal epithelial cells. We examined several chimeric MDESs in order to characterize the sequence differences responsible for the observed enzymatic activity differences between MDES1 and 2. However, chimeric MDESs exhibited very low Δ^4-desaturase and C-4 hydroxylase activities. It is likely that the overall

three-dimensional structure is essential for both activities, and therefore, the identification of particular domains responsible for enzymatic characteristics may not be possible. Thus, we focused on the 34-amino acid region of MDES212 between the first and second His-boxes and constructed chimeras with small differences in this region (Fig. 2A and B). This enabled us to identify a sequence essential for the C-4 hydroxylase activity of MDES2 and to search databases for DES2 gene family members also containing the XAFGY (X = T or A or V; Y = T or N) sequence.

We identified DES2 family members in pig, rat, chicken, zebrafish, and *Xenopus*, and DES1 family members in pig, rat, chicken, and zebrafish. Phylogenetic analyses demonstrated that the DES1 and DES2 families form independent groups that probably diverged after the evolution of vertebrates.

Several groups reported that His-boxes are essential for the catalytic activities of fatty acid and sphingolipid desaturases and hydroxylases [18–21]. Mizutani et al. reported that the DES2s of mouse and human have an additional conserved $HX_{2-3}HH$ motif, designated IIb, at their carboxyl termini [16]. We introduced point mutations in this additional His-box, HLPQHH, producing sequences such as DLPQHH, HLPQHY, and DLPQHY, and we examined the enzyme activities of the resultant molecules. We could not detect any changes in the Δ^4-desaturase and C-4 hydroxylase activities of these mutated MDES2s (data not shown). This additional His-box is conserved in mouse, rat, and human DES2 sequences, but not in chick, zebrafish, and *Xenopus* sequences (Fig. 3C). Based on these data, we conclude that this His-box has no effect on the C-4 hydroxylase activity of DES2.

Although the XAFGY region exists in human and zebrafish DES1 (Fig. 3B), these DES1 proteins exhibited C-4 hydroxylase activity lower than that of DES2 proteins from the same species (data not shown). The role of this region is unknown, but we speculate that it may become functional when coupled with DES2 His-boxes. We found that cytochrome b5 enhanced the C-4 hydroxylase activity of MDES2, but not its Δ^4-desaturase activity (unpublished data). Thus, this His-box region may be important in the stimulation by cytochrome b5. Another possibility is that this region produces three-dimensional structure important for C-4 hydroxylase activity in MDES2. In this study, we identified a region essential for hydroxylase activity, but further studies are needed to characterize the role of this sequence in C4-hydroxylation.

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